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Detection of mycoplasmas in cell cultures by PCR: a one year study

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Abstract

Over a one year period, 372 different cell cultures were received for the detection of mycoplasmas. PCR analysis of the 16S rRNA gene was compared to DNA fluorescence staining by DAPI (4',6-diamidine-2-phenylindole dihydrochloride). 278 samples (75%) were found to be negative using both methods, 86 samples (23%) were found positive with the PCR and the DAPI staining method, and 6 samples (2%) were found to be infected with other bacteria. The 86 positive samples were also subjected to specific amplification in order to identify the species. Only two of them (3.5%) could not be identified. In conclusion, PCR is a rapid and reliable method for detecting and identifying mycoplasmas in tissue cultures.

Key words: Cell cultures; Mollicutes; 16S rRNA gene amplification

1. Introduction

Mycoplasmas (the trivial name for the microorganisms of the Mollicutes class) are small ($\approx$ 0.5 μm) wall-less bacteria. Their presence in cell cultures can go undetected because of the absence of a cell wall and their adherence to the cells surface render them invisible to the naked eye. Contaminations of cell lines in the past occured through the use of animal sera contaminated with *Mycoplasma arginini* or *Mycoplasma hyorhinis* or *Acholaeplasma laidlawii* and through mouth pipetting from the manipulator source of *Mycoplasma orale, Mycoplasma fermentans, Mycoplasma salivarium* and *Mycoplasma pirum* [1,2]. Since better sterilization techniques have

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been enforced, the most current mode of transmission is due to contamination from cell line to cell line through aerosols. It is estimated that about 5 to 30% of the world cell lines are contaminated with mycoplasmas (5–16%, [1]; 5–87%, [2]; 25,7% [3]; 29% [4] and 23% (this paper)). Usually the mycoplasmas do not destroy cell lines but they do alter their metabolism so that they can affect the interpretation of the results of research studies (for reviews, see [1] and [2]). It is generally difficult and time consuming to cure a cell line so that it is best to monitor them from time to time and to check all the newly introduced cell lines from outside and keep them separate until tested to avoid cross-contamination [2]. Unfortunately, the methods commonly used to detect the presence of mycoplasmas are tedious, time consuming and necessitate a certain expertise. Therefore a faster (results in 10 h) and simple method based on PCR (polymerase chain reaction) was devised and tested for validation over a one year period on 372 different samples received for testing. The method was compared with the DAPI fluorescence staining assay (results in 7 days) and direct growth in conventional growth media (results in 10 days).

2. Materials and methods

2.1. Growth media

Base medium (BD): Difco PPLO broth 21 g, Difco tryptone broth 10 g, Difco yeast extract 5 g, distilled water to one litre. Autoclaved and kept at 4°C until use.

Glucose complete medium (BDG): 20 ml horse serum, 0.2 ml phenol red 1%, 1 ml ampicilline (66 mg/ml), 1 ml glucose 50% were added to 78 ml BD under sterile condition and the pH adjusted to about 7.8 with NaOH 1 M. Color is red.

Arginine complete medium (BDA): as above but with the glucose replaced by 5 ml of arginine hydrochloride 5% and the pH adjusted to about 7.2. Color is yellow.

2 ml of media were distributed in screw cap tubes. One tube containing BDG and one tube containing BDA were infected with 0.2 ml of cell suspension and the pH was monitored over a 10 day period. BDG becomes yellow with glucose fermenting mycoplasmas and BDA becomes purple with arginine hydrolyzing mycoplasmas.

2.2. DAPI staining of mycoplasma DNA

In order to amplify eventual mycoplasma contaminants, the cell lines to be tested were cultured and/or cocultured for three and six days with a 3T6 (ATCC-CCL96) sensitive fibroblast-like cell line, in tubes containing a cover-slip (Leighton). After culture in their usual medium (attached cell lines) or in Dulbecco's medium (cocultures) for 3 and 6 days in a CO_2 incubator, the coverslips were washed with PBS (20 mM phosphate, 150 mM NaCl, pH 7.5) and then fixed with Carnoy (ethanol 6 vol., chloroform 3 vol. and acetic acid 1 vol.) for 10 min and dried for 1 h at 37°C, rinced in PBS, stained with DAPI (0.1 μg/ml in PBS) for 15 min., rinsed 2 × with PBS and mounted on slides in PBS + 50% glycerol and observed under an epifluorescence microscope at 200–400 × magnification.

2.3. Preparation of samples for the PCR

1 ml of cellular suspension or 1 ml of scraped cells were centrifuged in a 1.5 ml Eppendorf tube for 15 min at maximum speed at 4°C. The pellets were resuspended in washing buffer (10 mM Tris-HCl pH 8.3, KCl 50 mM, $MgCl_2$ 1.5 mM) mixed well and re-centrifuged. The washed pellets can be kept at −20°C until treatment if necessary.

2.4. Treatment of the samples

The pellets were resuspended in 50 μl of solution A + B, incubated 1 h at 60°C, then 10 min at 95°C or in boiling water and kept at −20°C until PCR amplification (solution A: 10 mM Tris-HCl pH 8.3, KCl 100 mM, $MgCl_2$ 2.5 mM; solution B: 10 mM Tris HCl pH 8.3, $MgCl_2$ 2.5 mM, Tween 20 1%, Triton × 100 1% and proteinase K 120 μg/ml from a stock solution at 20 mg/ml in 5 mM Tris-HCl pH 7.8 plus 50% glycerol kept at −20°C).

2.5. DNA amplification by PCR

Oligonucleotide primers from Tables 1 and 2 were used to amplify the DNA of the samples. 40 μM primers 1 and 2 each, 40 μM pBRI and pBR2 or pBR6 each and 2 fg of pBR322 DNA were mixed in 50 mM KCl, 10 mM Tris-HCl pH 8.3, $MgCl_2$ 1.5 mM, autoclaved Difco gelatin 0.1%, $2.5.10^{-5}$ M trimethylammonium chloride (Aldrich Milwaukee, USA), 0.2 mM dNTP, 1 unit of Taq DNA polymerase and 2 drops of mineral oil. 2 μl DNA sample direct (reaction 1) and 2 μl DNA sample

Table 1
Primer names and sequences

Name of primer	Sequence 5′–3′ (Genbank)	Site on gene or plasmid
pBR1	CATCTCGGGCAGCGTTGGGT	1424–1443
pBR2	ACAAGCTGTGACCGTCTCCG	2143–2124
pBR6	AGCGCAGCGAGTCAGTGAGC	2389–2370
RNA5	AGAGTTTGATCCTGGCTCAGGA	10–31
RNA3	ACGAGCTGACGACAACCATGCAC	1068–1043
UNI	TAATCCTGTTTGCTCCCCAC	782–764
ACH3	AGCCGGACTGAGAGGTCTAC	277–296
ARG2	TCAACCAGGTGTTCTTTCCC	460–440
FER	AAGAAGCGTTTCTTCGCTGG	203–222
HYR	GAAAGGAGCTTCACAGCTTC	198–217
ORA	GGAGCGTTTCGTCCGCTAAG	199–218
PIR	GTCCGTTTGGACCGCTATAG	203–222
SAL	GCTGCGTCAACAGTTCTCTG	849–830
HOM	TGAAAGGCGCTGTAAGGCGC	193–212
PNEU-GEN	CCTGCAAGGGTTCGTTATTT	204–223

pBR1, pBR2, pBR6 from pBR322; all the other primer sequences were deduced from either the conserved regions of the 16S RNA for the mollicute determination: RNA3, RNA5 and UNI or the variable 16S RNA region [3] for the species specific determination: ACH3 (*A. laidlawii*), ARG (*M. arginini*), FER (*M. fermentans*), HYR (*M. hyorhinis*), ORA (*M. orale*), PIR (*M. pirum*), SAL (*M. salivarium*), HOM (*M. hominis*), PNEU-GEN (*M. pneumoniae* and *M. genitalium*).

Table 2
Primer use and length of amplified fragment in bp

Species	Primer 1	Primer 2	Length	Primer controls	Length
Mycoplasmas (all)	RNA5	RNA3	1058	pBR1 + pBR2	719
A. laidlawii	ACH3	UNI	505	pBR1 + pBR6	974
M. arginini	RNA5	ARG2	450	pBR1 + pBR6	974
M. fermentans	FER	UNI	579	pBR1 + pBR6	974
M. hyorhinis	HYR	UNI	584	pBR1 + pBR6	974
M. orale	ORA	UNI	582	pBR1 + pBR6	974
M. pirum	PIR	UNI	579	pBR1 + pBR6	974
M. salivarium	RNA5	SAL	838	pBR1 + pBR6	974
M. hominis	HOM	UNI	589	pBR1 + pBR6	974
M. pneumoniae + *M. genitalium*	PNEU	RNA3	864	pBR1 + pBR2	719

diluted 1/10 (reaction 2) to make 50 μl were added through the oil. The samples were put for 15 min at 95°C, then subjected to 30 cycles at 95°C 30 s, 58°C 1 min 30 s (or 64°C for specific species), 72°C for 1 min 30 s increased 1 s/cycle and a 10 min increase at 72°C for the last cycle, then incubated at 17°C until use.

2.6. Analysis of amplified DNA

20 μl of the amplified products were electrophoresed in Tris-borate buffer on 0.8% agarose gels. Each run had a positive control (*A. laidlawii* prepared from a culture in the same way as the cellular extracts and used at 2 different 1/10 dilutions), a negative control (3T6 cellular extracts used directly or at 1/10) and a molecular weight marker (phage λ DNA digested with *Hin*dIII enzyme). PCR products were visualized by ethidium bromide staining.

3. Results

3.1. Sensitivity of the PCR

DNA from *M. fermentans* was diluted serialy and used for amplification with primers RNA3 and RNA5 alone or together with pBR1 and pBR2 and pBR322 DNA (Fig. 1). A detectable product visible on stained agarose gels could be seen with dilutions representing $\leqslant$ 10 fg or the equivalent of about 10 mycoplasmas in 10^6 cells in presence of 3T6 DNA (lanes 2 to 10) and 1 fg or the equivalent of 1 mycoplasma in 10^6 cells in absence of 3T6 (lanes 12 to 19).

3.2. Detection of mycoplasmas in cell lines

Throughout the year 1992, our laboratory received 372 cell samples to test. Using RNA5 and RNA3 primers along with pBR1 and pBR2 primers as inhibition controls, 86 samples were found to be positive by both PCR and DAPI staining (23%), 278 were found to be negative by both PCR and DAPI staining (75%), 6 were found to be infected with other bacteria (2%). In Fig. 2, seven different samples were chosen to illustrate the necessity of using two successive dilutions for each sample

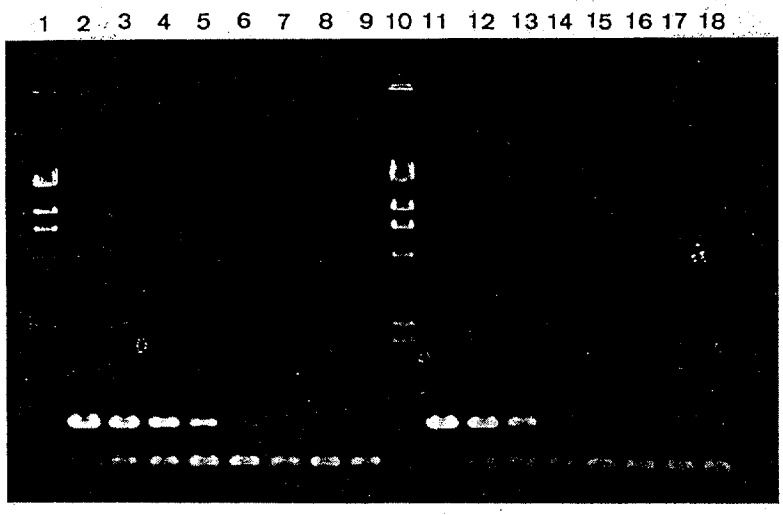

Fig. 1. Electrophoresis of PCR products from a serial dilution of *M. fermentans* DNA in the presence (lanes 2–8) and in the absence (lanes 11–17) of 3T6 DNA. All include pBR322 amplification product; negative controls (sterile distilled water instead of *M. fermentans* DNA) are in lane 9 and 18. Lanes 1 and 10 contain lambda *Hin*dIII fragments marker.

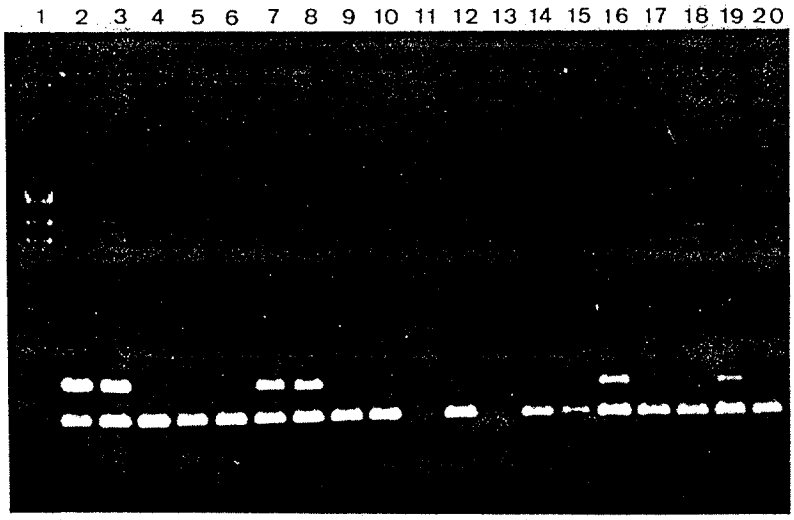

Fig. 2. Electrophoretic analysis of seven different DNA samples using RNA3 and RNA5 primers together with pBR322 DNA and pBRI and pBR2 primers. Lane 1: lambda *Hin*dIII DNA fragments; lanes 2 and 3: *A. laidlawii* positive control DNA at two one to ten dilutions; lane 4: pBR322 DNA and primers only; lanes 5 and 6: 3T6 negative control DNA at two one to ten dilutions; direct and one to ten dilutions of positive samples a (lanes 7,8), c (lanes 11,12), e (lanes 15,16), g (lanes 19,20) and negative samples b (lanes 9,10), d (lanes 13,14) and f (lanes 17,18).

Table 3
List of the mycoplasmas species identified, percentage and growth properties

Species	Number	Total %	Growth
M. hyorhinis	44	51.2	9/44 = 20%
M. orale	13	15.1	13/13 = 100%
M. fermentans	10	11.6	9/10 = 90%
M. arginini	10	11.6	10/10 = 100%
A. laidlawii	1	1.2	1/1 = 100%
Mixed infection	6	7.0	6/6 = 100%
Not identified	2	2.3	0/2 = 0%
M. pirum	0		
M. salivarium	0		
M. hyorhinis homini	0		
M. pneumoniae	0		
M. genitalium	0		

and an external positive control like pBR322 DNA (see Discussion below). Note for example that the first dilution of samples c (lane 11) and d (lane 13) showed no amplification of either pBR322 or sample DNA, sample e showed a positive amplification in the first dilution for pBR322 only (lane 15) and the second dilution of sample g (lane 20) was positive but to a much lesser extent.

3.3. Identification of the mycoplasmas found in the positive cell lines

We analyzed all the mycoplasmas from positive samples by PCR in order to determine to which species they belonged. The results are summarized in Table 3. Two samples could not be identified at all by either PCR or ELISA (Boehringer Mannheim Detection kit 1296 744; 2, av. du Vercors, BP 59, F 38242 Meylan Cédex) nor could they be grown.

Six were infected with a mixture of strains: 4 with *M. hyorhinis* + *M. arginini*, 1 with *M. hyorhinis* + *M. arginini* + *M. fermentans*, 1 with *M. arginini* + *M. fermentans*. Among the 44 *M. hyorhinis*, 35 (80%) could not be grown in conventional media (no gas pack) and 1 *M. fermentans* in 10 (10%) failed to grow. All the other species could be grown.

4. Discussion

PCR provides a simple and rapid alternative detection system to that of culture or DAPI staining. When we began this project over two years ago, we started to use the primers which were elaborated by Dr. A. Blanchard, one of which he had already used (RNA5) [6]. At that time we did not look for the highest specificity. RNA3 and RNA5 primers can also recognize some of the low GC gram + bacteria but of course no eucaryotic DNA. This seemed sufficient since infection of tissue cultures by bacteria can easily be spotted. Sensitivity is not a problem with tissue cultures. Most of the time cells are heavily contaminated; this has been shown by

scanning electron micrographs like in [1] and our unpublished results. Furthermore DAPI staining could not be interpreted if there were not at least 10 to 50 mycoplasmas per cell [2,7]. This is why in this study, DAPI and PCR results correlate 100%. Even if the PCR sensitivity for real infected cultures was lower than for mocked infected culture (Fig. 1) the sensivity would still be much higher for PCR than for DAPI staining. The real problem with PCR derives from the presence of inhibitors produced by some cell lines. This is the reason why a pBR322 positive control and two different sample dilutions were always included in the assays (see Fig. 2). In this way it was possible to detect all infected cultures except in one case where we had a positive DAPI and a negative PCR sample (one out of the 372 samples). Since that cell line had been left in the refrigerator, when we retested it two weeks later, it was still negative with PCR. But finally, three weeks later, when we wanted to see if we could improve the preparative procedure, the sample became positive by PCR regardless of the method used for the DNA preparation. We suggest that the inhibitor was finally destroyed during the three weeks in the refrigerator. PCR is therefore an excellent method to detect mycoplasmas but with the caveat that there is always a possibility of having a false negative.

The results obtained in our lab (Table 3) are not statistically significant since we often receive many cell lines from the same labs and most of the time if one line is contaminated by one particular mycoplasma there is a good chance that the others are too. The cell lines received for testing varied enormously in type and origin. PCR is the only reliable way to detect mycoplasmas especially in insect cell lines where the results of DAPI staining are particularly difficult to interpret.

Altogether PCR is as efficient as any other method used alone but it is more sensitive and much faster and should become the method of choice for detection of mycoplasmas in general.

5. Acknowledgements

We are indebted to Dr. A. Blanchard for the choice of the sequences of the primers RNA3 and RNA5 and we thank C. Prévost for skilled technical assistance.

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Rapid, Sensitive PCR-Based Detection of Mycoplasmas in Simulated Samples of Animal Sera

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A fast and simple method to detect mycoplasmal contamination in simulated samples of animal sera by using a PCR was developed. The following five mycoplasma species that are major cell culture contaminants belonging to the class *Mollicutes* were investigated: *Mycoplasma arginini*, *Acholeplasma laidlawii*, *Mycoplasma hyorhinis*, *Mycoplasma orale*, and *Mycoplasma fermentans*. After a concentration step involving seeded sera, genus-specific primers were used to amplify a 717-bp DNA fragment within the 16S rRNA gene of mycoplasmas. In a second step, the universal PCR was followed by amplification of variable regions of the 16S rRNA gene by using species-specific primers, which allowed identification of contaminant mycoplasmas. With this method, 10 fg of purified DNA and 1 to 10 color-changing units of mycoplasmas could be detected. Since the sensitivity of the assay was increased 10-fold when the amplification products were hybridized with an internal mycoplasma-specific ^{32}P-labelled oligonucleotide probe, a detection limit of 1 to 10 genome copies per PCR sample was obtained. This highly sensitive, specific, and simple assay may be a useful alternative to methods currently used to detect mycoplasmas in animal sera.

Mycoplasmas (the trivial name for microorganisms belonging to the class *Mollicutes*) are the smallest self-replicating bacteria. They are common and serious contaminants of cell cultures, and this remains one of the major problems encountered in biological research, in biological diagnosis, and in biotechnological production with cell cultures. This problem is actually widespread, and the incidence of contamination varies from 5 to 87% of cell cultures (3, 28, 31, 42) depending on the cell line, the test used, and the quality control practices used. The class *Mollicutes* is subdivided into more than 120 species (41), but only 20 species have been isolated from cell cultures (1, 2, 40). Of these 20 species, which have been isolated from bovine, human, and porcine sources, 5 (*Mycoplasma arginini*, *Acholeplasma laidlawii*, *Mycoplasma hyorhinis*, *Mycoplasma orale*, and *Mycoplasma fermentans*) account for 95% of all contaminations (1, 3, 25, 26). It has been shown that mycoplasmas produce a variety of effects on cultured cells. Besides affecting cellular growth and morphology, mycoplasmas are known to be able to alter amino acid and nucleic acid metabolism and immunological and biochemical properties and to induce chromosome aberrations, leading to experiments whose results are often unreliable (1, 3, 12, 24, 25). The main sources of contamination of clean cultures are mycoplasma-infected cultures (1). The origins of the contaminants (i.e., the direct sources) are often laboratory personnel (*M. orale* and *Mycoplasma salivarium* have been isolated from oropharynxes of 25 to 80% of healthy individuals; *M. fermentans* has been isolated more rarely) (38) and commercial animal sera used in culture media (*M. arginini*, *A. laidlawii*, *M. hyorhinis*) (1, 2).

Mycoplasma cells are pleomorphic, have diameters of 300 to 800 nm, and lack cell walls (41). Because of their small size and pliability, mycoplasmas can pass through the 450- and 220-nm-pore-size membrane filters usually used for cell culturing (17). When high-pressure filtration is used some mycoplasmas can occasionally penetrate 100-nm-pore-size final filters (5, 41). Like most cell cultures infected by mycoplasmas, serum infections are rarely detected by visual inspection or light microscopy in the absence of obvious signs of infection (e.g., turbidity of sera or media, pH changes, or cytopathic effects on cell lines) (42). Moreover, serum is not very propitious for mycoplasma growth since it lacks host cells and some essential nutrients and also contains toxic and inhibitory components (27). Low contamination rates and reduction of contamination rates by filtration for a long time made detection and isolation of mycoplasmas from commercial sera impossible (2, 5). Three methods are currently sensitive enough to detect mycoplasmas: microbiological culturing, adenosine phosphorylase (AdoP) screening, and the indicator cell culture technique. However, each method has certain disadvantages. The method based on specific broth or agar medium culturing is time-consuming (several weeks), some strains (especially *M. hyorhinis* strains) are difficult to grow (3, 15, 24, 42), and experience is needed to perform the technique and interpret the results (42). The biochemical method lacks specificity since some bacteria (e.g., *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*) produce nucleoside phosphorylase (4, 5, 16), while some mycoplasma species (e.g., *Mycoplasma pneumoniae* FH, *Mycoplasma pirum*, *Mycoplasma lipophilum*) produce practically none (16). Moreover, AdoP is a soluble enzyme that is active in the absence of live mycoplasmas (i.e., contaminants) (24). The method based on inoculation into mycoplasma-free indicator cell cultures is time-consuming because three passages and a final detection technique (e.g., DNA fluorescent staining with 4′,6-diamidino-2-phenylindole dihydrochloride [34] or bisbenzimidazole fluorochrome Hoechst 33258 [8] or an enzyme-linked immunosorbent assay [ELISA] with specific antibodies) are needed (19). Except for the ELISA, these methods cannot be used for direct identification of the contaminants.

In this paper we describe the development of a PCR-based method to detect mycoplasmas in simulated samples of animal sera. Our data show that this rapid and reproducible method possesses not only the high sensitivity necessary for detecting mycoplasmas in sera and the specificity necessary for identifying contaminant mycoplasmas (thus indicating possible

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sources), but also the simplicity necessary for easy, unambiguous interpretation of results.

MATERIALS AND METHODS

Growth media. The broth and agar media used for growth and titration were BDA, BDG, and GD, as described by Roulland-Dussoix et al. (33). The base medium contained 21 g of PPLO broth (Difco Laboratories, Detroit, Mich.), 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and enough distilled water to bring the volume to 1 liter. For BDG, 78 ml of autoclaved base medium was supplemented with 20 ml of horse serum, 0.2 ml of 1% phenol red, 66 mg of ampicillin per ml, and 1 ml of 50% glucose under sterile conditions, and the pH was adjusted to 7.8 with 1 M sodium hydroxide. For BDA, the base medium was supplemented as described above except that the glucose was replaced with 5 ml of 5% arginine hydrochloride and the pH was adjusted to 7.2. For GD, 1 g of agar was added to 80 ml of base medium and the preparation was autoclaved; the medium was completed by adding 20 ml of horse serum, 1 ml of 50% glucose, and 1 ml of ampicillin (66 mg/ml) under sterile conditions.

Strains and growth conditions. The strains used included *M. hyorhinis* BTS7^T (= ATCC 17981^T) (T = type strain), *M. orale* CH19299^T (= ATCC 23714^T), *M. fermentans* PG18^T (= ATCC 19989^T), and *A. laidlawii* PG8^T (= ATCC 23206^T). *M. arginini* G230^T was obtained from the Collection of the Institut Pasteur. Portions (0.2 ml) of late-exponential-phase mycoplasmas were grown on 2-ml portions of BDA and BDG at 37°C under microaerophilic conditions. Arginine-hydrolyzing mycoplasmas alkalinize BDA, and the yellow color of BDA changes to purple. Glucose-fermenting mycoplasmas acidify BDG, and the red color of BDG changes to yellow.

In addition, the following microorganisms were investigated to evaluate the specificity of the primers used for the PCR: *M. pirum*, *M. salivarium*, *M. pneumoniae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Mycoplasma pulmonis*, *Ureaplasma urealyticum*, *Staphylococcus pasteurii*, *B. subtilis*, *Enterococcus faecalis*, *Streptococcus* sp., *Clostridium difficile*, *Clostridium perfringens*, *Clostridium ramosum*, *Clostridium innocuum*, *Rhodococcus* sp., *Escherichia coli*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus flavus*.

DNA extraction. Genomic DNAs were extracted from mycoplasmas by the method described by Carle et al. (7) to evaluate PCR sensitivity. Following extraction, DNAs were purified by equilibrium centrifugation in CsCl-ethidium bromide continuous gradients as described by Sambrook et al. (36).

Sera and infection. Commercial mycoplasma-free tested sera from horses, newborn calves, and fetal calves were obtained from GIBCO-BRL, Cergy Pontoise, France. These sera were diluted five times in a physiological salt solution and infected with known amounts of broth-grown mycoplasmas, defined by determining the numbers of CFU and color-changing units (CCU) as described by Rodwell and Whitcomb (32). The CCU titration was based on the correlation between the growth of mycoplasmas and the color changes of broth media supplemented with appropriate pH indicators.

Treatment of seeded samples. Simulated samples (10 ml) were centrifuged in 13.2-ml Ultra-Clear tubes (Beckman Instruments, Palo Alto, Calif.) by using a type SW41 Ti rotor (Beckman Instruments) at 4°C for 30 min at 20,000 × *g*. The resulting pellets were resuspended in washing buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), and the resulting preparations were mixed well and centrifuged with an Eppendorf model 5414 instrument at 4°C for 15 min at the maximum speed. DNAs were released from the samples by adding 25 μl of solution A (10 mM Tris-HCl [pH 8.3], 100 mM KCl, 2.5 mM $MgCl_2$) and 25 μl of solution B (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 1% Tween 20, 1% Triton X-100, 120 μg of proteinase K [Appligene, Inc., Pleasanton, Calif.] per ml; the proteinase K was from a 20-mg/ml stock solution in 5 mM Tris-HCl [pH 7.8]–50% glycerol kept at −20°C) to the washed pellets, incubating the preparation for 1 h at 60°C, boiling it for 10 min, and then chilling it on ice.

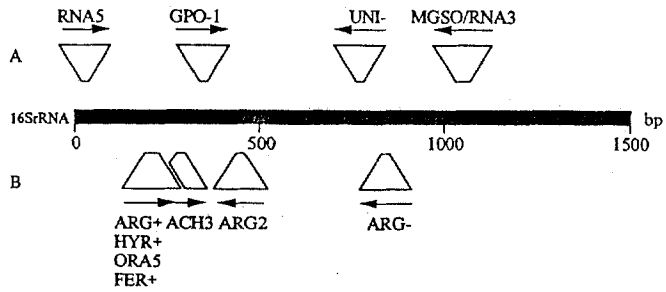

FIG. 1. Positions of the oligonucleotide primers used for detection of mycoplasmas at both the genus (A) and species (B) levels on a schematic physical map of the mycoplasmal 16S rRNA gene.

Oligonucleotide primers for the PCR and oligonucleotide probe. The existence of regions which exhibit sequence variability at the genus and species levels in mycoplasmal 16S rRNA genes allowed us to select genus- and species-specific primers for the PCR (Fig. 1 and Tables 1 and 2). A 40-pmol portion of radioactive DNA probe GPO-1 (43) was labelled in 25 μl of a solution containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 200 μCi of [γ-^{32}P]ATP (Amersham International, Amersham, England), and 30 U of T4 polynucleotide kinase (United States Biochemical Corp., Cleveland, Ohio) by incubating the preparation for 45 min at 37°C. The enzyme was inactivated by heating the preparation for 5 min at 65°C. The 5′-end-labelled oligonucleotide was purified on a Nick column (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions.

DNA amplification. To avoid contamination by naturally occurring DNA or by PCR product carryover, we used separated areas for PCR preparations and products. We routinely autoclaved and used UV to irradiate reagents that could be autoclaved and irradiated without affecting their performance.

TABLE 1. Sequences of the primers used in this study

Primer[a]	Sequence	Location (positions)
RNA5	5′-AGAGTTTGATCCTGGCTCAGGA-3′	10-31
RNA3	5′-ACGAGCTGACGACAACCATGCAC-3′	1043-1065
GPO-1	5′-ACTCCTACGGGAGGCAGCAGTA-3′	338-359
MGSO	5′-TGCACCATCTGTCACTCTGTTAACCTC-3′	1029-1055
UNI−	5′-TAATCCTGTTTGCTCCCCAC-3′	763-782
ARG+	5′-GTGAAAGGAGCCCTTAAAGC-3′	193-212
ARG2	5′-TCAACCAGGTGTTCTTTCCC-3′	440-459
ARG−	5′-CTGCGTCAGTGAACTCTCCA-3′	829-848
HYR+	5′-GAAAGGAGCTTCACAGCTTC-3′	198-217
ORA5	5′-GGAGCGTTTCGTCCGCTAAG-3′	199-218
FER+	5′-AAGAAGCGTTTCTTCGCTGG-3′	203-222
ACH3	5′-AGCCGGACTGAGAGGTCTAC-3′	277-296

[a] RNA5, RNA3, GPO-1 (43), MGSO (43), and UNI− are genus-specific primers. ARG+, ARG2, and ARG− (*M. arginini*), HYR+ (*M. hyorhinis*), ORA5 (*M. orale*), FER+ (*M. fermentans*), and ACH3 (*A. laidlawii*) are species-specific primers.

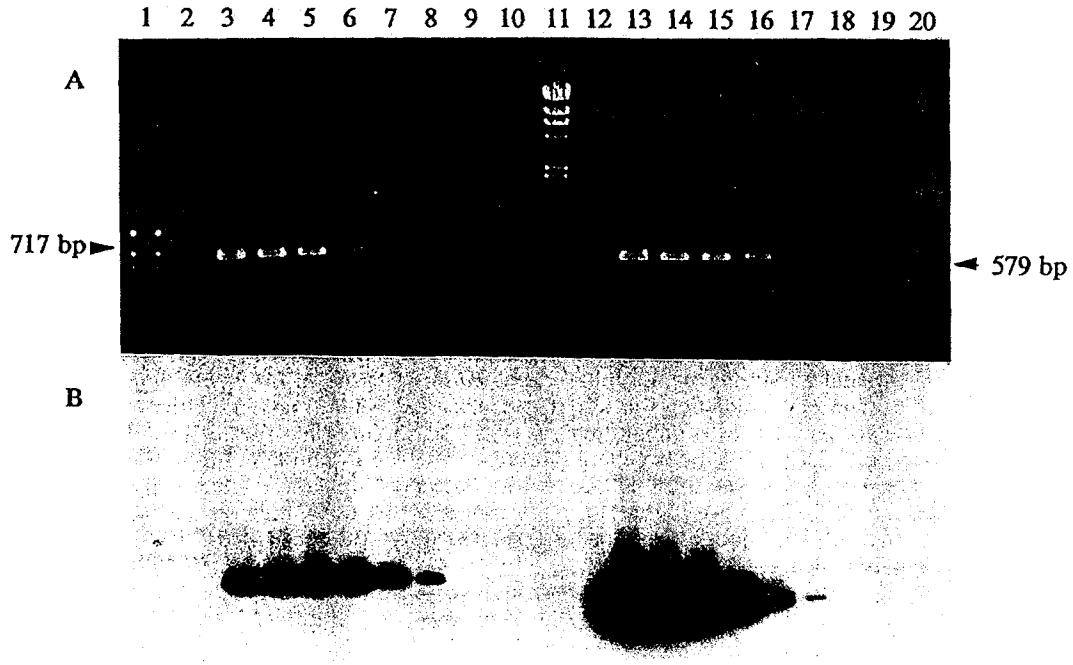

FIG. 2. Sensitivity of the PCR detection assay. Purified DNA from *M. fermentans* was serially diluted and subjected to a PCR with genus-specific primers (lanes 2 to 10) or with species-specific primers (lanes 12 to 20). (A) Ethidium bromide staining of DNA on the agarose gel. (B) Southern blot analysis of the gel shown in panel A, using [γ-^{32}P]ATP-labelled probe GPO-1. Lanes 1 and 11, DNA markers (lane 1, ϕX174 replicative-form DNA *Hin*cII digest [Pharmacia]; lane 11, λ DNA *Hin*dIII digest); lanes 2 and 12, negative controls (sterile distilled water); lanes 3 and 13, 100 pg; lanes 4 and 14, 10 pg; lanes 5 and 15, 1 pg; lanes 6 and 16, 100 fg; lanes 7 and 17, 10 fg; lanes 8 and 18, 1 fg; lanes 9 and 19, 100 ag; lanes 10 and 20, 10 ag.

teuri, *Escherichia coli*, *Rhodococcus* sp., *C. perfringens*), and even with yeasts (e.g., *Saccharomyces cerevisiae*). The specificity of the species-specific primer sets was also investigated. At an annealing temperature of 64°C, no amplification product was detected when mycoplasmal DNA from a species other than the species tested or nonmycoplasma bacterial DNA was examined (see Materials and Methods for the species tested). Use of primer sets specific for *M. arginini*, *A. laidlawii*, *M. hyorhinis*, *M. orale*, and *M. fermentans* resulted in amplification of DNA fragments having the expected sizes (449, 505, 584, 583, and 579 bp, respectively). Tests with purified DNA were then completed by performing specificity studies with simulated samples. The primer sets exhibited the same specificity with all five type strains mentioned in Materials and Methods and with cell culture contaminant strains identified in our laboratory (13 *M. arginini* strains, 3 *A. laidlawii* strains, 46 *M. hyorhinis* strains, 13 *M. orale* strains, and 12 *M. fermentans* strains).

In order to enhance the specificity of our method, we used the three-primer PCR described by Kai et al. (20). We used RNA3, RNA5, and inner primer UNI− for the genus-specific PCR and ARG+, ARG−, and inner primer ARG2 for the species-specific PCR for *M. arginini* (Table 1). The reaction produced an additional band having the predicted size (772 bp in the genus-specific PCR and 266 bp in the PCR specific for *M. arginini*), which confirmed the identity of the initial PCR product (1,055 bp in the genus-specific PCR and 655 bp in the PCR specific for *M. arginini*). The detection limit with this technique was also 10 fg (Fig. 3).

DISCUSSION

Contamination of animal sera by mollicutes is a widespread problem that has biological and economic importance for cell culturists and serum processors. Since there is at present no reliable, reproducible, fast, sensitive, specific assay for detecting mycoplasma infections in animal sera, our aim was to develop a procedure which is superior to classical microbiological culturing, AdoP activity screening, and indicator cell culture techniques. In this paper we describe the use of the PCR in a mycoplasma infection detection system for animal sera that is useful in laboratory and industrial applications.

The exquisite sensitivity of our PCR-based detection tech-

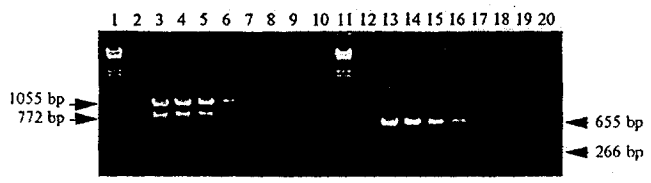

FIG. 3. Sensitivity of the three-primer PCR detection assay. Purified DNA from *M. arginini* was serially diluted and subjected to the universal PCR by using three genus-specific primers (lanes 2 to 10) or to the specific PCR by using three species-specific primers (lanes 12 to 20). Lanes 1 and 11, DNA markers (*Hin*dIII-digested λ DNA); lanes 2 and 12, negative controls (sterile distilled water); lanes 3 and 13, 100 pg; lanes 4 and 14, 10 pg; lanes 5 and 15, 1 pg; lanes 6 and 16, 100 fg; lanes 7 and 17, 10 fg; lanes 8 and 18, 1 fg; lanes 9 and 19, 100 ag; lanes 10 and 20, 10 ag.

plasmas in animal sera. Compared with the time-consuming and fastidious indicator cell culture and broth-agar culture detection methods and the enzymatic detection method, which is not very specific, the PCR assay is a promising method, for the diagnosis of animal serum contamination by mycoplasmas. The method described above could provide an interesting alternative to the currently used detection methods. The viability and rapidity of the assay could be useful for cell culturists. Moreover, in the serum supply industry, this assay could be combined with a confirmation test to provide quality control procedures for animal serum producers, to improve the validity of results, and to reduce the time of quarantine. Although the results obtained with simulated samples described above are encouraging, the suitability of the assay for detection and identification of mycoplasmas in commercial animal sera remains to be established. Therefore, we are currently performing additional studies with a large number of commercial samples in order to validate the PCR assay.

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ACTA VET. BRNO, 62, 1993: 49–53

STAINING WITH AN ACRIDINE ORANGE DERIVATIVE FOR THE DETECTION OF MYCOPLASMAS IN CELL CULTURES

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Abstract

Fischer O., Dagmar Zendulková: *Staining With an Acridine Orange Derivative for the Detection of Mycoplasmas in Cell Cultures.* Acta vet. Brno, 62, 1993: 49–53.

24 cell cultures were examined for the presence of mycoplasmas by fluorescence methods using 3-amino-6-methoxy-9-(2-hydroxyethylamino) acridine (AMHA) or bisbenzimide 33258 (Hoechst), and by culture in liquid media containing glucose or arginine and, under anaerobic conditions, on solid media. Mycoplasmas were detected in 17.9, 25.5 and 28.6 per cent of the cultures by staining with AMHA, staining bisbenzimide and by culture, respectively. The differences in sensitivities of the methods were not significant. Disadvantages of staining with AMHA were discussed.

Fluorescence, staining, mycoplasma contamination

Detection of mycoplasmas in cell cultures by fluorescence methods is based on the visualization of nucleic acids of mycoplasmas. The stains used include bisbenzimide (Chen 1977), olivomycin (Mikhailova et al. 1982) and 4'-6-diamidine-2-fenylindol (DAPI) (Russell et al. 1975). Jayat-Vignoles et al. (1990) described the use of a new acridine orange derivative — 3-amino-6-methoxy-9-(2-hydroxyethylamino) acridine (AMHA) — for this purpose.

In our laboratory, checks of the presence of mycoplasmas in cell cultures have been performed using the culture and the bisbenzimide 33258 (Hoechst) methods. Staining with AMHA was examined to extend the set of the detection methods, and the results were compared with those of the bisbenzimide and the culture methods.

Materials and Methods

The examined cell cultures (Table 1) came from the cell culture bank and other laboratories of the Veterinary Research Institute, Brno, as well as from laboratories outside the institute. Altogether 117 samples of 24 cell cultures, of which 23 were monolayer cultures and 1 (myeloma line FO) was a semisuspension culture, were examined.

The monolayers were grown in a closed system in Mueller, Legroux or Roux flasks in Eagle's Minimal Essential Medium supplemented with 10 per cent of fetal calf serum, penicillin (100 I.U. per 1 ml) and streptomycin (100 μg per 1 ml).

The cells were released enzymatically before re-seeding them by a solution containing 0.1 to 0.2 per cent of chymotrypsin or trypsin, and 0.02 per cent of versene.

The propagation of hepatoma cell lines was described by Hankinson (1979).

Before the examination by fluorescence, the cells were inoculated into test tubes containing pieces of slides (5 × 20 mm) and 2 ml of growth medium, and incubated at 37 °C.

A cell suspension density was chosen that would not produce a complete monolayer during 3 to 5 days of growth. The propagation of the semi-suspension myeloma cell line FO and its co-culture with Vero cells, used as an indicator, were described earlier (Fischer et al. 1991).

The acridine orange derivative AMHA was kindly supplied by Prof. H. W. Zimmermann from the Institut für physikalische Chemie der Universität in Freiburg, FRG.

Stock solution of AMHA in distilled water (10 mM) was stored in the dark at +4 °C and working solutions were prepared before staining. The concentration 5 μM and an exposure period

Visible colonies of mycoplasmas developed on solid media after 14 days of incubation under anaerobic conditions.

Staining with bisbenzimide or AMHA visualized mycoplasmas as minute, intensively fluorescent, yellow-green particles in the vicinity of large, intensively fluorescent, yellow-green nuclei (Fig. 2 and 4); only fluorescent cell nuclei were observed in mycoplasma-free cell cultures (Fig. 1 and 3).

The two staining methods differed in their intensities and persistences of fluorescence.

While nucleoli were clearly distinguishable in the fluorescent nuclei, the background was dark and cytoplasma was stained in sporadic cells and only very weakly in cell cultures stained with bisbenzimide, no nucleoli were recognisable and the background and cytoplasma were bright yellow-green in those stained with AMHA (Fig. 1 to 4).

The fluorescence persisted longer in the bisbenzimide-stained than in the AMHA-stained cell cultures.

Discussion

It follows from the description of the two fluorescence methods that staining with AMHA is more time-consuming than that with bisbenzimide (45 vs. 25 minutes).

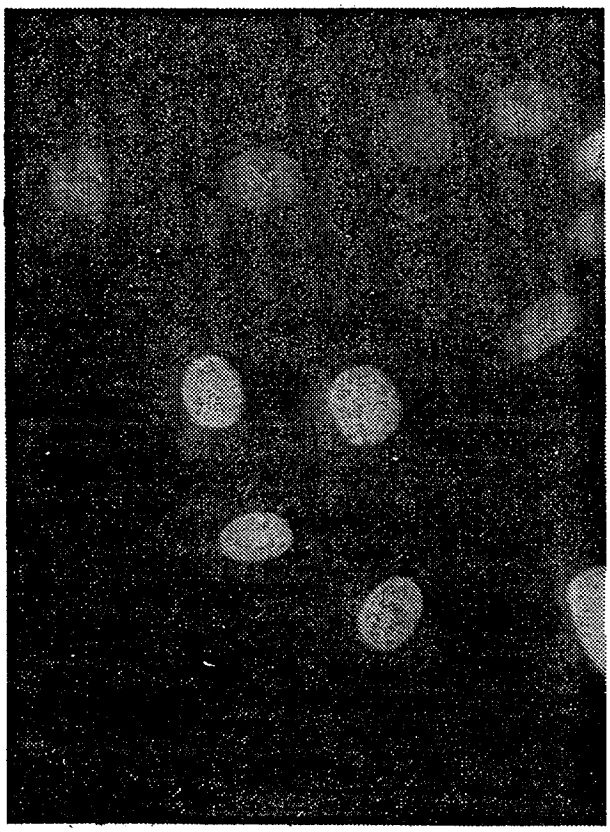

Fig. 1: Mycoplasma-free Vero cells. Stained with bisbenzimide.

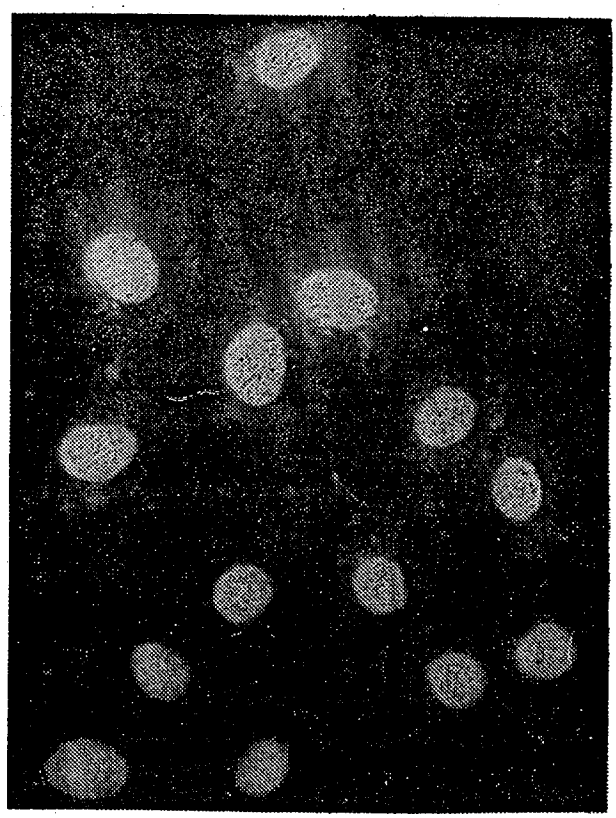

Fig. 2: Mycoplasma-infected Vero cells. Stained with bisbenzimide.

Použití derivátu akridinové oranže k detekci mykoplazmat v buněčných kulturách

Ve 24 buněčných kulturách byla zjišťována přítomnost mykoplazmat fluorescenčními metodami s barvením 3-amino-6-methoxy-9-(2-hydroxyethylamino) akridinem (AMHA) nebo bisbenzimidem 33258 (Hoechst) a kultivací v tekutých mediích s glukózou nebo argininem a kultivací na pevných půdách v anaerobních podmínkách.

Barvením AMHA byla mykoplazmata zjištěna v 17,9 % kultur, barvením bisbenzimidem v 25,5 % kultur a kultivačními metodami v 28,6 % kultur.

Rozdíly v citlivosti použitých metod nebyly statisticky významné.

Применение производной акридинового оранжа для определения микоплазмы в клеточных культурах

В 24 клеточных культурах определяли наличие микоплазмы флюоресцентными методами с окраской 3-амино-6-метокси-9-(2-гидроксиэтиламино) акридином (АМНА) или бисбензимидом 33258 (Хэхст) культивированием в жидких средах с глюкозой или аргинином и культивированием на прочных почвах в анаэробных условиях.

Окраской АМНА была микоплазма выявлена в 17,9 % культур, окраской бисбензимидом - в 25,5 % культур и методами культивирования - в 28,6 % культур.

Разница чувствительности примененных методов не отличалась статистической значимостью.

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Polymerase chain reaction-based diagnosis of mollicute infection of commercial animal sera

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Abstract

A total of 72 commercial animal sera were assayed for mollicute infection by a polymerase chain reaction (PCR)-based detection method and by four classical detection methods. The methods included microbiological assay by inoculation onto agar and into broth, DNA staining of an indicator cell line with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) fluorochrome, enzyme-linked immunosorbent assay (ELISA) and adenosine phosphorylase (AdoP) screening. Mollicutes were detected in 2 of the 72 sera assayed. The species isolated was *Acholeplasma laidlawii* in both cases. When detection methods were compared, PCR, microbiological culture and DNA staining were perfectly concordant. ELISA and AdoP detection produced false-negative results for 1 of the 2 infected sera each. False positive results appeared in 1 of the 70 mollicute-free sera with ELISA and in 16 of the 70 with AdoP detection. It was concluded that the PCR-based detection method is a useful tool in addition to current methods of detection of mollicutes in animal sera, with regard to reliability, sensitivity, specificity and time.

Key words: Polymerase chain reaction; Mollicute; Animal serum

1. Introduction

Mollicute contamination of cell lines is a widespread problem in biological research, biological diagnosis and biotechnological production using cultured cells. Despite the increasing awareness of cell culturists during the past decade, mollicute infection remains a common occurrence in cell cultures [1–5]. It has been shown that mollicutes produce a variety of effects on host cells, thus influencing the significance and validity of virtually every experimental result [2,6–9].

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The main origins of mollicute infection of cell cultures are mollicute infected cultures, laboratory personnel (*Mycoplasma orale, Mycoplasma fermentans*) and commercial animal sera used in cell culture media (*Mycoplasma arginini, Acholeplasma laidlawii, Mycoplasma hyorhinis*) [6,10]. Therefore, filtration of cell culture media through membrane filters of 450 and 220 nm porosity is a common practice. While filtration steps at this level usually suffice, some mollicutes can occasional penetrate final filters of 100 nm under high pressure filtration, due to their small size and pliability [4,11,12]. Therefore, there is a requirement for routine, periodic screening of sera for mollicutes.

Serum infection by mollicutes is rarely detected with the naked eye, in the absence of obvious signs of infection (e.g., turbidity). Unlike cell cultures, serum is not very propitious to mollicute growth, since it lacks host cells and some essential nutrients and also contains toxic and inhibitory components [13]. Due to small contamination rates and their reduction by filtration, attempts to detect and isolate mollicutes from commercial sera have for a long time failed [10,11]. Several sensitive tests among those developed to detect mollicutes in cell cultures have been adapted to diagnose mollicute infection of sera (e.g., microbiological culture method, DNA fluorescent staining, ELISA and AdoP screening) [10,11]. However, each method shows certain disadvantages.

This report describes an evaluation of a PCR-based method for the detection of mollicutes in animal sera. In addition, the method is compared to four other detection assays, including broth-agar microbiological culture, DNA fluorescent staining with DAPI, ELISA with specific antibodies and AdoP screening. Data presented indicate that this rapid assay can be used as a sensitive and specific system for detection and identification of mollicutes in commercial animal sera.

2. Materials and methods

2.1. Sera

The 72 final lots of sera to be examined included 62 lots of fetal bovine sera, 4 lots of newborn calf sera, 4 lots of calf sera and 2 lots of horse sera. These sera had previously been filtered and controlled for lack of bacterial, fungal and mollicute contamination by processors. However, some lots were turbid and so were sequentially filtered through 450, 220 and 100 nm Nalgene filters to remove or reduce the bacterial and fungal contamination before the sera could be tested for mollicutes. A part of the initial serum and of each filtrate was saved and tested.

Serum lots were kindly provided by Gibco BRL Life Technologies (Cergy Pontoise, France), Laboratoires Eurobio (Les Ulis, France), France Biochem (Meudon, France), Bayer Diagnostics (Puteaux, France), Hyclone Laboratories Inc. (Logan, UT, USA) or obtained indirectly via various laboratories of the Institut Pasteur (Paris, France) from other suppliers including ATGC, Biological Industries, Cytosystems, DAP, Dominique Dutscher, Institut Jacques Boy, J Bio/Techgen, Labsystems and SeraLab.

2.2. Mollicute detection methods

PCR assay

In the first part of the assay, mollicutes were concentrated by centrifugation. Serum samples (35 ml) were centrifuged in 38.5 ml Ultra-Clear tubes (Beckman Instruments, Palo Alto, CA, USA) using a SW28 rotor (Beckman Instruments), for 30 min at 20 000 *g* at 4°C. The pellets were washed and DNA released as described by Roulland-Dussoix et al. [14]. Briefly, the pellets were resuspended in 25 μl of solution A (10 mM Tris HCl (pH 8.3)–100 mM KCl–2.5 mM $MgCl_2$) and 25 μl of solution B (10 mM Tris HCl (pH 8.3)–2.5 mM $MgCl_2$–1% Tween 20–1% Triton X–100 and 120 μg/ml proteinase K (Appligene Inc., Pleasanton, CA, USA), incubated 1 h at 60°C then boiled for 10 min and chilled on ice. DNA amplification by PCR was performed essentially as described by Dussurget and Roulland-Dussoix [15]. Genus-specific primers described by Van Kuppeveld et al. [16] were used to amplify the DNA of each sample (Table 1). Species-specific primers selected in regions of mycoplasmal 16S rRNA genes which present a high extent of interspecies sequence variability, were used to identify the contaminant mollicutes of infected sera (Table 1). A 10 μl DNA sample was incubated in a 100 μl reaction volume containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 25 μM tetramethylammonium chloride (Aldrich, Milwaukee, WI, USA), 80 pmol of primer 1 and of primer 2, 200 μM each dNTP and 2U of *Taq* polymerase (Amersham International, Buckinghamshire, UK). The reaction mixture was overlaid with 2 drops of mineral oil. The DNA sample (direct and one to ten diluted) was added last, through the oil. Amplification was carried out in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The sample was subjected to an initial denaturation step 15 min at 95°C then to 30 cycles at 95°C for 30 s, 58°C for 1 min 30 s (genus-specific PCR) or 64°C for 1 min 30 s (species-specific PCR), 72°C for 1 min 30 s increased 1 s per cycle. Finally, the sample was subjected to an additional extension step 10 min at 72°C. Twenty μl of the PCR product were analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Negative controls (distilled sterile water) were included in each step of the experiment (concentration, extraction, amplification). A positive control (purified mollicute DNA, direct and one to ten diluted) was also included in each set of amplification.

Microbiological culture

Broth and agar media used for cultivation were BDA, BDG and GD. The base medium was made of 21 g PPLO broth (Difco, Detroit, MI, USA), 10 g tryptone (Difco), 5 g yeast extract (Difco) and distilled water to one liter. For BDG, 78 ml of the autoclaved base medium were completed with 20 ml horse serum, 0.2 ml phenol red 1%, 1 ml ampicillin 66 mg/ml and 1 ml glucose 50%, under sterile conditions and pH was adjusted to 7.8 with sodium hydroxide 1 M. For BDA, the base was completed as above except glucose was replaced by 5 ml of arginine hydrochloride 5% and the pH adjusted to 7.2. For GD, 1 g of agar was added to 80 ml of base and autoclaved. The medium was completed with 20 ml of horse serum, 1 ml of glucose 50% and 1 ml of ampicillin 66 mg/ml under sterile conditions. Mollicutes were

Table 1
Use, sequence and position of genus- and species-specific primers and length of amplified fragments

PCR specificity	Primers			Length of amplified fragment (bp)
	Name	Sequence (5′–3′)	Position*	
Mollicutes	GPO1	ACTCCTACGGGAGGCAGCAGTA	338–359	717
	MGSO	TGCACCATCTGTCACTCTGTTAACCTC	1039–1055	
M. arginini	RNA5	AGAGTTTGATCCTGGCTCAGGA	10–31	449
	ARG2	TCAACCGGTGTTCTTTCCC	440–459	
A. laidlawii	ACH3	AGCCGGACTGAGAGGTCTAC	277–296	505
	UNI-	TAATCCTGTTTGCTCCCCAC	763–782	
M. hyorhinis	HYR+	GAAAGGAGCTTCACAGCTTC	198–217	584
	UNI-	TAATCCTGTTTGCTCCCCAC	763–782	
M. orale	ORA5	GGAGCGTTTCGTCCGCTAAG	199–218	583
	UNI-	TAATCCTGTTTGCTCCCCAC	763–782	
M. fermentans	FER+	AAGAAGCGTTTCTTCGCTGG	203–222	579
	UNI-	TAATCCTGTTTGCTCCCCAC	763–782	
M. pirum	PIR+	GTCCGTTTGGACCGCTATAG	203–222	579
	UNI-	TAATCCTGTTTGCTCCCCAC	763–782	
M. salivarium	RNA5	AGAGTTTGATCCTGGCTCAGGA	10–31	839
	SAL-	GCTGCGTCAACAGTTCTCTG	830–849	
M. hominis	HOM+	TGAAAGGCGCTGTAAGGCGC	193–212	589
	UNI-	TAATCCTGTTTGCTCCCCAC	763–782	
M. pneumoniae	PNEU+	CCTGCAAGGGTTCGTTATTT	204–223	861
	RNA3	ACGAGCTGACGACAACCATGCAC	1043–1065	
M. genitalium	PNEU+	CCTGCAAGGGTTCGTTATTT	204–223	861
	RNA3	ACGAGCTGACGACAACCATGCAC	1043–1065	

*Position is relative to *Escherichia coli* 16S rDNA nucleotide sequence.

concentrated by centrifugation as mentioned above. The pellets were resuspended in 100 μl physiological salt solution. For each sample, 4 agar dishes (55 mm) and 4 broth tubes (2 ml liquid medium) of each medium were used. After concentration of the sample, 50 μl were inoculated onto each agar dish and 50 μl were added to each broth tube. Two dishes and 2 tubes of each medium were incubated aerobically at 37°C. Two dishes and 2 tubes of each medium were incubated anaerobically using the BBL GasPak system (Becton Dickinson and Co., Cockeysville, MD, USA) at 37°C. After 7 days incubation, the broths were subcultivated onto GD agar (50 μl

per dish) for final enrichment. Formation of characteristic colonies on agar and indicator colour shift of media were monitored during a month before regarding cultures as negative. In each step of enrichment, and in each condition of incubation, a dish and a broth tube of each medium were inoculated with 50 μl physiological salt solution (negative controls).

Indirect DAPI DNA staining

In order to amplify the level of mollicute infection, the serum to be tested was added to culture medium (instead of 5% mollicute-free serum) of a mollicute-free indicator cell line, 3T6-Swiss albino mouse embryo fibroblasts (ATCC CCL96). After 3 passages with the test medium, the presence of mycoplasma was confirmed by DNA staining as described by Roulland-Dussoix et al. [14]. Briefly, each inoculated 3T6 cell line was cultured in 2 Leighton tubes containing a glass coverslip and incubated for 3 and 6 days at 37°C in an atmosphere of 5% CO_2–95% air. After washing, cells were fixed with ethanol–chloroform–acetic acid (6:3:1, v/v/v) for 10 min, and dried for 1 h at 37°C. The fixed preparation was stained for 15 min with DAPI (0.1 μg/ml in PBS) and mounted. Microscopic slides were examined under an epifluorescence microscope at 200–400 × magnification for the presence of typical extranuclear fluorescent particles. Cell line inoculated with culture medium containing mollicute-free serum was run in each experiment as negative control.

ELISA

Serum sample was first enriched by incubation with 3T6 indicator cell line for 3 passages, as described above. The presence of mollicute was detected by ELISA using specific polyclonal antibodies against *M. arginini*, *A. laidlawii*, *M. hyorhinis* and *M. orale* from the Mycoplasma Detection Kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions. For each sample and each species, determination was done in duplicate, and negative and positive controls were included.

AdoP detection

The adenosine phosphorylase activity was evaluated by the microtechnique according to Bonissol et al. [17]. Briefly, 5 μl serum sample were incubated at 37°C for 3 days with 15 μl of substrate [8-^{14}C]adenine in the presence of α-D-ribose-1-phosphate, bovine serum albumin and Tris HCl-EDTA (pH 7.4). Five μl of marker containing adenine and adenosine were added at the end of the reaction. Then adenine and adenosine were separated by ascendant thin layer chromatography in distilled water. Two μl of the reaction mixture were spotted on Avicel cellulose foil (Schleicher and Schuell, Dassel, Germany). After 15 min of migration at 37°C, the spots were localized under UV light (254 nm), cut out and counted in a liquid scintillation counter. Each sample was screened in duplicate. Negative controls (distilled water and mollicute-free serum) were included in each set of experiment.

2.3. Analytical method

In most comparative studies, broth-agar culture is used as the reference test [18]. However, no technique can be regarded as the 'gold standard'. Hence, the results found positive (or negative) by at least 3 techniques were arbitrarily considered to be true positive (or negative). The assessment of positivity or negativity of the results found by other techniques was determined by comparison with the data from the 3 aforesaid concordant techniques as the reference

3. Results

3.1. Incidence of infection and identification of species

A total of 72 lots of commercial sera were tested for mollicute contamination. Of these, two sera were slightly turbid and one contained microparticules. They were all three contaminated by bacteria, but no mollicute was found. After 450 and 220 nm filtrations (see Materials and methods), bacteria were no longer there, but mollicutes were present in one filtrate. The three 100 nm filtrates were all found to be bacteria-free. In addition, 3 limpid sera were found to be contaminated by non-mollicute bacteria. Thus, 6 of 72 lots were contaminated by non-mollicute bacteria. The incidence of mollicute infection as shown by at least 3 methods was 2/72, including the lot contaminated by both mollicutes and other bacteria mentioned above. The 2 mollicute-positive lots came from the same supplier. The 7 mollicute and/or bacteria-positive lots all came from 4 of the 14 suppliers.

The 2 mollicute infected sera were shown to contain *Acholeplasma laidlawii*. The 6 bacteria infected sera were shown to be contaminated by *Staphylococcus* ssp. and *Flavobacterium* ssp.

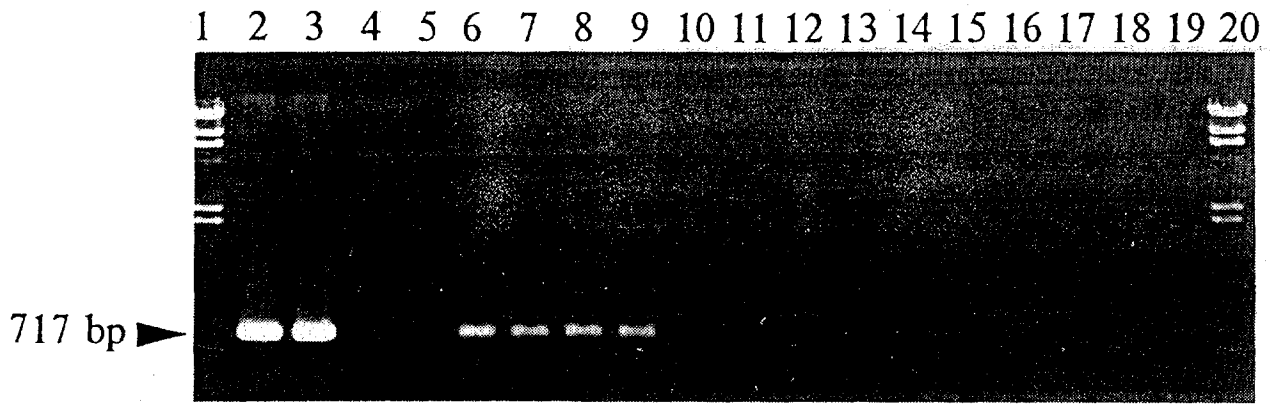

Fig. 1. Detection of mollicutes by PCR in commercial animal sera. Electrophoretic analysis of seven samples, direct and one to ten diluted in distilled sterile water, after amplification using GPO1 and MGSO primers. Lanes: 1 and 20; molecular weight markers: *Hin*dIII-digested λ DNA; 2, 3, *A. laidlawii* DNA, pure and 1:10 diluted (positive controls); 4, 5, distilled sterile water (respectively, extraction and amplification negative controls); 6–9, positive samples; 10–19, negative samples.

3.2. Individual results of mollicute detection assays

PCR assay

Two of the 72 sera were found to be mollicute-positive in universal conditions PCR, i.e., using genus-specific primers (Fig. 1). The 2 samples were analyzed in specific conditions PCR, i.e., using species-specific primers. Both contaminants were identified as *Acholeplasma laidlawii.*

Microbiological culture

Using the broth-agar assay, two of the 72 sera tested were found to be infected with mollicutes. The two mollicute strains were isolated on GD from culture and from subculture but only in BDG and aerobic conditions. Four of the 72 sera were found to be contaminated by non-mollicute bacteria.

Indirect DAPI DNA staining

Two sera were found to be unequivocally infected with mollicutes. The one was diagnosed after 3 days incubation, the other after 6 days incubation. Five of the 72 sera were found to be infected with non-mollicute bacteria.

ELISA

Two sera were found to be infected with mollicutes. The first lot was contaminated with *Acholeplasma laidlawii.* The second gave a positive signal with all mollicute species tested (*M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*).

AdoP detection

Seventeen of the 72 sera showed enzymatic activity. Five of the 17 were contaminated by non-mollicute bacteria.

3.3. Comparative results of mollicute detection assays

A comparison of the data from the five assays (Table 2) showed that the results were concordant in 54 (all negative) out of 72 samples. Positive results were discordant. Nevertheless, broth-agar, PCR and indirect DAPI assays showed a perfect

Table 2
Detection of mollicute infection in 72 commercial animal sera

Number of lots	Results of test				
	Culture	PCR	Indirect DAPI	ELISA	AdoP
54	–	–	–	–	–
15	–	–	–	–	+
1	–	–	–	+	+
1	+	+	+	–	+
1	+	+	+	+	–

concordance regarding the results. They were considered as the references. Two sera were found positive by all 3 methods. Sixteen false positive results and one false negative result were found by AdoP detection. One false positive and one false negative results were found by ELISA.

4. Discussion

Despite many improvements in quality control and detection procedures, mollicute infection of cell cultures remains a widespread problem of biological and economic importance. The infection is often introduced by the use of contaminated animal sera in culture media. Since all the techniques used to detect such contaminations possess inherent disadvantages with regard to reliability, reproducibility, time, cost, sensitivity and specificity, our aim was to develop an assay which is superior to detection techniques currently used. This paper describes the suitability of a PCR assay for the detection and identification of mollicutes in commercial animal sera, comparatively to microbiological culture, DNA staining, ELISA and AdoP detection.

The low incidence of mollicute infection in commercial sera tested was expected since serum processing and quality assurance procedures are constantly being improved. However, the two mollicute contaminated sera came from the same supplier and the 7 bacteria contaminated sera all came from only 4 of the 14 suppliers. Serum collection and processing varied from one company to another, suggesting a large quality differential depending on serum source, manufacturing and sterilization procedures. This was correlated to the heterogeneity of regulations, codes of quality and safety depending on the country and to the large price scale as reported by Hodgson [19,20].

The characterization of two strains of *Acholeplasma laidlawii* revealed a true infection of the fetal bovine sera, since the natural habitat of the species is bovine oropharynx and bovine genitourinary tract [6]. Moreover, *A. laidlawii* has rarely been isolated from oropharynx of healthy individuals [21], i.e., contamination by the laboratory personnel was very unlikely. Our findings were in agreement with others in the literature. *A. laidlawii* was isolated from 3 of the 131 final processed lots of commercial fetal bovine sera examined by Barile and Kern [10].

Infection of sera with *Staphylococcus* ssp. and *Flavobacterium* ssp. was not highly surprising since some serum processors subject blended serum lots to only 220 nm final filtration under pressure, which can force the smallest bacteria through the pores. However, laboratory personnel can not be ruled out as a possible source of contamination in the case of *Staphylococcus* ssp.

PCR was compared to four methods for the detection of mollicutes in sera. The small number of samples tested and the even smaller number of true positives must be emphasized in order to avoid misinterpretation of the results. PCR results were all in agreement with the tests results of the broth-agar assay and the indirect DAPI DNA staining. A comparison of the data from ELISA and PCR showed that the results were concordant in 70 cases with 2 discordant outcomes (one positive, one

negative). The false positive serum in ELISA, infected with bacteria, was probably due to a cross-reaction although no case was reported by the manufacturer of the kit. The false negative serum, slightly contaminated by mollicutes, was possibly due to a lack of sensitivity of the immuno-enzymatic assay [5,22]. A comparison of the data from AdoP detection and PCR showed that the results were concordant in 55 cases and discordant for 16 true negative and 1 true positive samples. Five of the 16 false positive sera were infected with bacteria which could possess AdoP [11,17,23]. The other 11 false positive sera, free from contaminants, could contain soluble AdoP, active in the absence of living mollicutes or bacteria [7]. The false negative could be explained by the presence of AdoP inhibitors in the serum tested or by the presence of mollicutes with low AdoP activity [23].

In our study, the PCR assay was at least as sensitive and specific as microbiological culture and DAPI staining. It was also more sensitive and specific than ELISA (1 false negative, 1 false positive), more sensitive and much more specific than AdoP detection (1 false negative, 16 false positives). These data were in agreement with our previous findings [15]. With the exception of the immuno-enzymatic detection kit, limited to four species, specific PCR assay was the only approach allowing a direct identification of the contaminant. Some mollicute species are difficult to grow on broth-agar media, in particular, *M. hyorhinis*, which is known to be a porcine and bovine sera contaminant [4,7,24]. Others have a low AdoP activity, e.g., *M. pneumoniae* and *M. pirum* [7,23]. All these species were detected by our specific PCR assay. PCR assay is also faster than all other tested methods. The results are available within a day, unlike 3 days for AdoP, 4 weeks for microbiological culture and 3–4 weeks for cell culture-based methods. PCR assay is less fastidious than culture-based methods, especially cell culture-based methods which necessitate the use of a permanent indicator cell line. Moreover, interpretation of the results is easier for PCR than for DNA staining and microbiological culture, since these latter rely on subjective reading and require training and experience.

Nevertheless, the rather expensive PCR assay can not be regarded as the 'gold standard' either. Unlike culture-based methods, the PCR doesn't differentiate between dead and viable mollicutes. Therefore, it could lead to elimination of sera containing dead mollicutes. It is also a constraining assay, since strict procedures must be taken to avoid contamination and to limit the risk of false positive results [25–28].

From these results, though limited to 72 samples, it can be concluded that the PCR assay provides a rapid, sensitive and reliable method for detecting and identifying mollicutes in commercial animal sera. In comparison with the PCR assay, the indicator cell culture and broth-agar culture-based detection methods are time consuming, fastidious and require experience. The immuno-enzymatic assay is relatively low sensitive and the AdoP detection method is poor specific. Thus, the PCR method described here seems to be promising for the diagnosis of animal sera contamination by mollicutes. The PCR, the broth-agar and the indirect DAPI DNA staining assays apparently detected all infections in our study. However, none of them can be taken as the absolute reference. Therefore, the use of at least two of these methods is still recommended for mollicute detection. Thus, the PCR assay could provide an

additional tool to present methods, rather than an alternative, useful for cell culturists, serum processors and suppliers.

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PROPOSED VICH PROCEDURE FOR THE DETECTION OF MYCOPLASMA CONTAMINATION

General procedure for detecting mycoplasma contamination

Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam sterilized or irradiated should be tested for the absence of mycoplasmas. Solid and liquid media such as Hayflick's, Frey's, or the 113.28 Heart Infusion mycoplasma media, as well as an indicator cell line, such as African green monkey kidney (VERO) cells, should be used to detect mycoplasma contamination. These media's and cell lines should be able to detect small numbers of test organisms including *Acholeplasma laidlawii* (ATCC # 23206), *Mycoplasma arginini* (ATCC #23838), *M. hyorhinis* (ATCC # 17981), and *M. orale* (ATCC # 23714). For avian biologicals, the test organism *M. synoviae* (ATCC # 25204) should also be used. The nutritive properties of each lot of solid medium should have CFUs within one standard deviation of the established count for each frozen or lyophilized lot of the above listed test organisms. An appropriate color change should occur in the liquid media when approximately 20 CFUs of each test organism are inoculate. Stained micro colonies of mycoplasma should be visible on the indicator cell at the endpoint dilution's established for each of the above listed test organisms.

One sample of each batch of vaccine should be tested. Inoculate one agar plate for each final batch of vaccine with 0.1 ml of the sample being tested and inoculate 100 ml of the selected liquid medium with 2 ml of the sample. Incubate the plates from final batches of vaccine at 35-37°C aerobically (an atmosphere of air containing 4-6 % CO_2 and adequate humidity) for 10-14 days. On day 7 and 14 after inoculation, subculture 0.1 ml from the liquid media onto 1 agar plate each. Incubate each plate aerobically for 10-14 days. If MSV, MCS, or ingredient of animal origin is being tested, inoculate 2 agar plates each of at least 2 selected media with 0.1 ml of the sample being tested. Incubate all plates at 35-37°C, and for each media incubate one aerobically and the second anaerobically (an atmosphere of nitrogen containing 5-10 % CO_2 and adequate humidity) for 10-14 days. Inoculate 2 ml of each sample into 100 ml of each of the selected broth media. Inoculate 0.05 ml of the sample into 2 wells of chambered slides (example; Lab-Tek) containing the indicator VERO cells and incubate aerobically for 3-5 days. On day 7 and 14 after inoculation, subculture 0.1 ml from each of the 2 liquid media onto 2 agar plates of the 2 selected media. Incubate 1 plate of each selected media aerobically and 1 plate anaerobically for 10-14 days. Incubate the inoculated liquid media at 35-37°C and observe periodically throughout the 14 days of incubation and if any color change occurs, subculture immediately.

Interpretation of mycoplasma test results

At the end of each 10-14 day incubation period examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma colonies has not occurred on any of the inoculated media. If at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or is broken, the test is invalid and needs to be repeated. If mycoplasma colonies are found on any agar plate, the test should be repeated once to confirm the mycoplasma contamination. If mycoplasma colonies are found on any of the agar plates of the retest, the test sample should be considered unsatisfactory because of mycoplasma contamination. After 3-5 days of incubation the VERO cell indicator chamber slides should be stained with a DNA fluorochrome stain (example; Hoechst Bisbenzamid), and if any of the sample chambers have micro colonies when examined microscopically this is a positive presumptive test for mycoplasma contamination, which must be confirmed. If the agar(s) used with this presumptive positive test does not show mycoplasma colonies the test needs to be repeated using additional formulations of mycoplasma media, and PCR in order to confirm mycoplasma contamination that was non cultivable on the first agar(s) used.

Points for discussion:

1. Are two incubation conditions (aerobic and anaerobic) necessary when testing final batches of vaccine?
2. Which media are acceptable for final product, MSV, MCS, and ingredient testing; Hayflick's, Frey's, Frii's, 9CFR 113.28, ATCC, M-96, etc.?
3. Should there be 1 standard media or a choice of 2 or more media?
4. Should exact formulations for media be specified in the standard method, or is it sufficient to require the growth of the test organisms?
5. Does each lab need to initially compare the growth promotion of their test organisms against an international set of titered test organisms? If yes then how often, yearly?
6. Should the testing of new lots of raw ingredients for growth promotion be required?
7. Which media are acceptable for poultry; vaccine, MSV, and MCS testing?
8. Does DPN-cysteine need to be required in media used for poultry vaccine testing?
9. Should T-strain testing be required on final batches, MSV, MCS, and/or ingredients of animal origin?
10. Should the broth inoculum be 1 ml, 2 ml or 10 ml?
11. Should the inoculum onto the agar be 0.1, 0.2 or 0.25 ml?
12. Would there be antibiotic (Gentamicin) inhibition of mycoplasma growth with the larger 10 ml inoculum?
13. Should PCR be used as a screening, confirmatory or final test?
14. Should there be different testing requirements for MSV, MCS, and final batches?
15. Should there be a list of media and production ingredients which need to tested for mycoplasma contamination?
16. Should the standard method call for repetitive looking at the same plates for 28 days or multiple plates for each subculture which can be looked at and thrown over the course of the 28 days of the test?
17. Should the agar plates be examined throughout the 28 days (at 3, 7, 10, 14, 21 days) or just on the 28th day of the test?
18. Should there be a requirement to test killed viral products for mycoplasma contamination before they are inactivated?
19. Should the indicator cell/DNA stain procedure be included as a standard procedure for non cultivable mycoplasma detection? What tests should be used to confirm noncultivable mycoplasma?
20. How will this mycoplasma standard setting VICH committee judge whether the adding of a new test or additional media is cost effective?

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